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Characterization of the Metabolic Flux and Apoptotic Effects of *O*-Hydroxyl- and *N*-Acyl-modified *N*-Acetylmannosamine Analogs in Jurkat Cells*

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The supplementation of the sialic acid biosynthetic pathway with exogenously supplied *N*-acetylmannosamine (ManNAc) analogs has many potential biomedical and biotechnological applications. In this work, we explore the structure-activity relationship of ManNAc analogs on cell viability and metabolic flux into the sialic acid biosynthetic pathway to gain a better understanding of the fundamental biology underlying "glycosylation engineering" technology. A panel of ManNAc analogs bearing various modifications on the hydroxyl groups as well as substitutions at the *N*-acyl position was investigated. Increasing the carbon chain length of ester derivatives attached to the hydroxyl groups increased the metabolic efficiency of sialic acid production, whereas similar modification to the *N*-acyl group decreased efficiency. In both cases, increases in chain length decreased cell viability; DNA ladder formation, Annexin V-FITC two-dimensional flow cytometry assays, caspase-3 activation, and down-regulation of sialoglycoconjugate-processing enzymes established that the observed growth inhibition and toxicity resulted from apoptosis. Two of the panel of 12 analogs tested, specifically Ac_4ManNLev and Ac_4ManNHomoLev, were highly toxic. Interestingly, both of these analogs maintained a ketone functionality in the same position relative to the core monosaccharide structure, and both also inhibited flux through the sialic acid pathway (the remainder of the less toxic analogs either increased or had no measurable impact on flux). These results provide fundamental insights into the role of sialic acid metabolism in apoptosis by demonstrating that ManNAc analogs can modulate apoptosis both indirectly via hydroxyl-group effects and directly through *N*-acyl-group effects.

The term "sialic acid engineering" refers to a technique where non-natural *N*-acetylmannosamine (ManNAc)[1] analogs intercept the sialic acid biosynthetic pathway and are incorporated into cellular sialoglycoconjugates in the place of sialic acid residues (Fig. 1) (1, 2). The impetus behind this strategy is to mimic nature, which uses >50 different forms of sialic acid to modulate the structure and function of sialic acid-bearing glycoproteins and lipids (3). By using synthetic *N*-acyl-modified ManNAc analogs, the surfaces of living cells can be endowed with novel properties not found in nature (4) that, depending on the exact analog used to perform this "submolecular microsurgery" (5), have the potential to elicit a variety of changes in the behavior of the host cell.

Theoretically, the ability to modify the cell surface and recombinant sialoglycoconjugates with molecular precision has the potential to regulate any biological process governed by sialic acid, such as cell growth and differentiation, communication among different cells, recognition of soluble factors, and attachment to, or disengagement from, the extracellular matrix (6). In practice, sialic acid engineering methods have already been demonstrated to regulate cellular responses ranging from adhesion to proliferation (7, 8) and have shown promise for use in biomedical applications such as inhibition of viral binding (9), modulation of the immune system (10), and selective delivery of diagnostic (11) or therapeutic agents (2) to cancer cells. ManNAc analogs can also modify the expression of polysialic acid (12, 13), a linear polysaccharide composed of entirely of α-2,8-linked sialic acid, which is implicated in the complex neural processes (14), synaptic plasticity (15, 16), and tumor metastasis (17). In addition to research and medical applications, ManNAc analogs also hold potential for biotechnological applications, such as increasing the product quality of recombinant sialoglycoconjugates (18). In addition to sialic acid biosynthesis, other glycosylation pathways have been targeted with exogenous sugar analogs; for example, acetylated *N*-acetyllactosamine derivatives have been used to modulate sialyl Lewis X expression toward inhibiting the metastatic potential of cancer cells (19, 20), and *N*-acetylgalactosamine (GalNAc) analogs have been used to replace GalNAc residues within cellular glycoconjugates (21).

Despite the many exciting potential applications of glycosylation-targeting metabolic engineering strategies, several challenges must be overcome before large-scale adoption of this technology to "real-world" applications becomes commonplace. A major challenge, the inefficient metabolic utilization of sugar analogs by cells, has been addressed by the development of acetylated monosaccharides (22–25). In a previous study, we demonstrated that various acetylated ManNAc analogs are used with up to 900-fold increased efficiency compared with

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[1] The abbreviations used are: ManNAc, *N*-acetylmannosamine; GalNAc, *N*-acetylgalactosamine; PI, propidium iodide; PS, phosphatidylserine; GNE, UDP-GlcNAc 2-epimerase/ManNAc 6-kinase.

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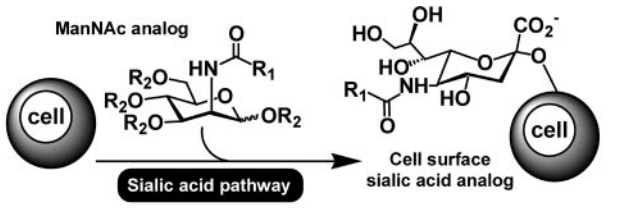

R_1 = 1 ManNAc	6 ManNLev	R_2 = –H	ManNAc
2 ManNProp	7 ManNHomoLev	$-COCH_3$	Ac_4ManNAc
3 ManNBut	8 ManNOxoHex	$-COCH_2CH_3$	$Prop_4$ManNAc
4 ManNPent	9 ManNOxoHept	$-CO(CH_2)_2CH_3$	But_4ManNAc
5 ManNHex	10 ManNOxoOct		

FIG. 1. **Overview of sialic acid engineering (*top*) and ManNAc analogs (*bottom*) used in this study.**

their free monosaccharide counterparts (26); a comparable increase in efficiency has been reported for acetylated disaccharides (24, 27, 28). The increase in uptake efficiency when the hydroxyl groups of a sugar are masked by acetyl esters led us to now investigate whether further elongation of the ester groups, resulting in even more hydrophobic compounds, would further increase the metabolic efficiency of analog utilization.

A downside to the increased metabolic efficiency of hydroxyl-derivatized analogs is that they inhibit growth and decrease cell viability under certain conditions; these factors threaten to diminish the widespread use of monosaccharide analogs bearing hydrophobic modifications on their hydroxyl groups. To devise general strategies to increase the safety of these efficiently used sugar analogs, the molecular and cellular bases of the growth inhibition and toxicity caused by these compounds require detailed investigation. In this study, we focused on sugars used in "sialic acid engineering" methods by probing the structure-activity relationship of ManNAc analogs that feed into the sialic acid pathway by investigating a panel of compounds bearing various modifications on the hydroxyl groups as well as different substitutions at the *N*-acyl position. These studies lay the groundwork for better understanding the molecular basis of this toxicity and for exploring a safe strategy to deliver potentially toxic sugar analogs into the glycosylation pathways.

Another aspect of this work is the discovery of new insights into the role of sialic acid in apoptosis. Results of this work show that non-natural ManNAc analogs behave as typical chemical toxicants that initiate and execute apoptosis in human cells. But, as a confounding factor, an ever-growing body of evidence shows that sialic acid itself plays key roles in apoptosis (6, 29–31); ManNAc analogs, therefore, by modulating metabolic flux into the sialic acid pathway, have the potential to evoke synergistic or antagonistic effects that either amplify or diminish that apoptotic response. To illustrate this point, 2 of the 12 analogs tested (Ac_4ManNLev and Ac_4ManNHomoLev) were highly toxic; interestingly, these two analogs were the only compounds to inhibit metabolic flux into the pathway. These results, discussed in more detail later in this report, demonstrate that ManNAc analogs, in addition to the glycosylation engineering applications discussed previously can also be exploited as research tools to gain new insights into the underlying biological basis of the connection of sialic acid metabolism to apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents, including RPMI 1640, Dulbecco's phosphate-buffered saline, and penicillin/streptomycin solution, were purchased from Sigma. Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Mannosamine hydrochloride and ManNAc were obtained from Pfanstiehl (Waukegan, IL); chemical reagents used in the synthesis of ManNAc analogs were purchased from Aldrich; organic solvents as well as copper sulfate, resorcinol, and periodic acid were from EM Sciences (Gibbstown, NJ). Annexin V-FITC assay kit for apoptosis detection and Caspase-3/CPP32 Colorimetric Assay kit for caspase-3 activity assay were purchased from MBL Co., Ltd. (Watertown, MA).

Synthesis of ManNAc Analogs—The synthesis, purification, and characterization of several of the ManNAc analogs used in this work followed procedures published previously. Specifically, Ac_4ManNAc, Ac_4ManNProp, Ac_4ManNBut, Ac_4ManNPent, Ac_4ManNHex, Ac_4ManNLev, Ac_4ManNOxoHex, Ac_4ManNOxoHept, and Ac_4ManNOxoOct were synthesized by the methods of Jacobs and co-workers (23). Ac_4ManHomoLev, $Prop_4$ManNAc, and But_4ManNAc are compounds that are previously unreported and are synthesized as described below.

General Procedure for the Preparation of $Prop_4$ManNAc and But_4ManNAc—To a stirred solution of ManNAc monohydrate (0.53 g, 2.2 mmol) in pyridine (2.0 ml) at 21 °C was added the corresponding anhydride (15.6 mmol) and 4-(dimethylamino)pyridine (cat.). After 24 h, the mixture was concentrated under vacuum and co-concentrated with toluene (25 ml). The residue was dissolved in methylene chloride (100 ml), washed with cold aqueous HCl (0.5 N, 100 ml), water (100 ml), and saturated $NaHCO_3$ (100 ml). The organic layer was filtered and concentrated. Column chromatography of the residue (hexanes/ethyl acetate) on silica gel provided the corresponding per-acyl compounds in the form of syrups that crystallized upon standing.

2-Acetamido-2-deoxy-1,3,4,6-tetra-O-propanoyl-α,β-D-mannopyranose ($Prop_4$ManNAc)—(1.0 g, 99%); R_f 0.3 (hexanes:ethyl acetate, 1:1); NMR ($CDCl_3$) (400 MHz) ^{1}H-NMR: δ (mixture of anomers, α/β ~10/90) 6.03 (d, 0.1H, J = 1.6), 5.87 (d, 0.9H, J = 2.0), 5.78 (d, 1H, J = 9.1), 5.34 (dd, 0.1H, J = 10.2, J = 4.6), 5.18 (t, 0.1H, J = 10.0), 5.13 (t, 0.9H, J = 9.6), 5.07 (dd, 0.9H, J = 9.8, J = 3.8), 4.75 (ddd, 0.9H, J = 9.2, J = 3.8, J = 1.8), 4.62 (ddd, 0.1H, J = 9.3, J = 4.4, J = 1.8), 4.29 (dd, 0.9H, J = 12.4, J = 5.4), 4.26 (dd, 0.1H, J = 11.3, J = 5.2), 4.11 - 4.07 (m, 1H), 4.03 (m, 0.1H), 3.81 (ddd, 0.9H, J = 9.2, J = 5.3, J = 2.4), 2.43 - 2.20 (m, 8H), 2.06 (s, 2.7H), 2.05 (s, 0.3H), 1.20 - 1.05 (m, 12H); ^{13}C-NMR (100 MHz): δ 173.9, 173.3, 173.2, 171.8, 171.1, 170.4, 169.9, 91.6, 90.6 ($^1J_{C1\text{-}H1}$ = 166), 73.5, 71.1, 68.7, 65.2, 65.1, 61.7, 60.4, 49.6, 49.4, 27.4, 27.3, 27.2, 23.3, 21.0, 14.2, 9.0, 8.9, 8.7, 8.5; FAB-MS m/z 468 [(M + Na)$^+$]; anal. calcd. for $C_{20}H_{31}NO_{10}$: C, 53.92; H, 7.01. Found: C, 53.89; H, 7.10.

2-Acetamido-1,3,4,6-tetra-O-butanoyl-2-deoxy-α,β-D-mannopyranose (But_4ManNAc)—(0.94 g, 87%); R_f 0.4 (hexanes:ethyl acetate, 2:1); NMR ($CDCl_3$) (400 MHz) ^{1}H-NMR: δ (mixture of anomers, α/β ~ 10/90) 6.03 (d, 0.1H, J = 1.7), 5.87 (d, 0.9H, J = 1.6), 5.76 (d, 1H, J = 9.3), 5.34 (dd, 0.1H, J = 10.4, J = 4.6), 5.18 (t, 0.1H, J = 10.2), 5.13 (t, 0.9H, J = 9.8), 5.06 (dd, 0.9H, J = 9.9, J = 4.0), 4.75 (ddd, 0.9H, J = 9.1, 3.8, 1.7), 4.63 (ddd, 0.1H, J = 9.3, J = 4.4, J = 1.9), 4.27 (dd, 0.9H, J = 12.4, J = 5.4), 4.23 (dd, 0.1H, J = 12.6, J = 5.2), 4.09 (dd, 0.9H, J = 12.4, J = 2.4), 4.05 (m, 0.1H), 4.01 (m, 0.1H), 3.80 (ddd, 0.9H, J = 9.4, J = 5.5, J = 2.4), 2.39 - 2.14 (m, 8H), 2.07 (s, 3H), 1.70 - 1.53 (m, 8H), 0.99 - 0.87 (m, 12H); ^{13}C-NMR (100 MHz): δ 173.0, 172.5, 172.3, 170.9, 170.7, 170.4, 91.5, 90.5 ($^1J_{C1\text{-}H1}$ = 165), 73.5, 71.1, 70.3, 68.6, 65.0, 61.8, 61.6, 49.6, 49.4, 35.8, 35.7, 23.3, 18.3, 18.2, 18.0, 17.9, 13.6, 13.5, 13.5, 13.4; FAB-MS m/z 524 [(M + Na)$^+$]; anal. calcd. for $C_{24}H_{39}NO_{10}$: C, 57.47; H, 7.84. Found: C, 57.35; H, 7.77.

1,3,4,6-Tetra-O-acetyl-N-(4-oxo-hexanoyl)-D-mannosamine (Mixture of Anomers) (Ac_4ManNHomo-Lev)—To a solution of 2.6 ml (19 mmol) of triethylamine in 56 ml of anhydrous tetrahydrofuran was added 2.47 g (19 mmol) of 4-oxo-hexanoic acid (Sigma). The reaction was stirred for 15 min at room temperature under a N_2 atmosphere, after which 2.4 ml (19 mmol) of isobutyl chloroformate was added dropwise by a syringe. The reaction was stirred for 3.0 h, during which time a white precipitate formed. The 4-oxo-hexanoic acid carbonic anhydride was used in the next step without further purification. To a solution of 3.6 g (17 mmol) of mannosamine hydrochloride in 112 ml of 1:1 H_2O/tetrahydrofuran was added 3.1 ml (22 mmol) of triethylamine. The solution was stirred for 15 min at room temperature, after which the 4-oxo-hexanoic acid carbonic anhydride was added dropwise by an addition funnel. The reaction was stirred for another 36 h under a N_2 atmosphere, and the solution was concentrated *in vacuo*. The crude compound was acetylated by treatment with 40 ml of 2:1 Pyr/Ac_2O. The reaction was stirred for 12 h at room temperature, and then the solution was concentrated *in vacuo*. The resulting syrup was washed with 1.0 M HCl (2 × 30 ml) and saturated $NaHCO_3$ (1 × 30 ml) and then dried over Na_2SO_4. Purification of the crude compound by silica gel chromatography yielded a white foam.

^{1}NMR ($CDCl_3$) (500 MHz) ^{1}H-NMR: δ 6.36 (d, 1H, J = 9.3), 6.25 (d, 1H, J = 9.1), 6.01 (app d, 1H, J = 1.7), 5.83 (app d, 1H, J = 1.8), 5.28 (dd, 1H, J = 10.1, J = 4.5), 5.15 (app t, 1H, J = 10.1), 5.09 (app t, 1H, J = 9.5), 5.01 (dd, 1H, J = 9.7, J = 4.1), 4.72 (ddd, 1H, J = 9.2, J = 4.0, J = 1.8), 4.58 (ddd, 1H, J = 9.3, J = 4.4, J = 1.8), 4.27 (dd, 1H, J = 12.4,

J = 5.3), 4.26 (dd, 1H, J = 12.4, J = 4.8), 4.12 (dd, 1H, J = 12.4, J = 2.6), 4.06 (dd, 1H, J = 12.4, J = 2.4), 4.01 (ddd, 1H, J = 10.0, J = 4.6, J = 2.4), 3.78 (ddd, 1H, J = 9.3, J = 5.1, J = 2.7), 2.82 - 2.71 (m, 4H), 2.63 - 2.56 (m, 2H), 2.52 - 2.41 (m, 6H), 2.15, 2.13, 2.12, 2.11, 2.04, 2.03, 1.98, 1.97 (8 s, 3H each), 1.06 (t, 3H, J = 7.3), 1.05 (t, 3H, J = 7.3); ^{13}C-NMR (125 MHz): δ 210.8, 210.5, 173.1, 172.6, 170.2, 169.7, 168.7, 168.4, 91.9, 90.8, 73.5, 71.4, 70.3, 69.2, 65.6, 65.5, 62.2, 62.1, 49.3, 37.6, 37.5, 36.1, 30.2, 30.1, 21.0, 21.0, 20.9, 20.9, 20.8, 8.0; HR-MS (FAB$^+$) calcd. for $C_{20}H_{29}LiNO_{11}$ [(M + Li)$^+$] 466.1741; Found 466.1904.

Cell Culture—Jurkat (a human T lymphoma-derived line) cells were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were maintained at 37 °C in a humidified air atmosphere with 5% CO_2.

Measurement of Cell Counts and Viability—Cell counts were measured using a hemocytometer and a Coulter model Z2 cell counter. Depending on the experiment, the viable cells were detected by using either the trypan blue exclusion test, where dead cells, which absorb trypan blue, can be identified under an optical microscope, or through propidium iodide (PI) staining coupled with analysis by flow cytometry (32). Cell viability was defined by the ratio of the viable cell number to the total cell number. Lethal dose values for the panel of analogs evaluated in Fig. 9 were determined as described previously (26).

Measurement of Sialic Acid Production—The production of sialic acid in cells incubated in the presence of ManNAc analogs was determined by adaptation of the periodate resorcinol assay (23, 33) originally described by Jourdian and co-workers (34). For the experiments monitoring a time-scale change of production of sialic acid in cells, cells were seeded at the same density of 1.0×10^6 cells/ml into a six-well plate. ManNAc analogs were added at the same concentration of 500 μM, and the culture was incubated for defined time periods. During incubation, aliquots of cells were collected and resuspended in 200 μl of phosphate-buffered saline on a daily basis (up to 4 days) for the data shown in Figs. 2 and 3, at the end of 5 days for the data shown in Fig. 10*B*, and after 18 h for the experiment shown in Fig. 10*C*. In all cases, cells were counted immediately after harvesting, and lysates were made by subjecting the cells to freeze-thaw cycles followed by periodic acid oxidation, treatment with hydrochloric acid and copper sulfate resorcinol, and quantitation by A_{630} readings, as described (34) to obtain values for total sialic acid. When sialic acid content is given on a single cell basis, the number of cells used for this determination is always the final number of cells, determined immediately before lysis (and not the seeding density at the start of the experiment).

Apoptosis Assays

Culture Conditions for Treatment of Cells with Analogs—In this study, unless otherwise specified, toxicity experiments were performed by using sugar analog concentrations of 500 μM and initial cell densities of 1.00×10^6 cells/ml. These conditions were selected because they were shown previously to support maximal levels of metabolic flux through the sialic acid pathway for Ac_4ManNAc while largely avoiding toxic and growth inhibitory effects to cells (26). These conditions, however, were close to the boundary where growth inhibition began to be manifest; therefore, all other analogs, which proved to be more toxic than Ac_4ManNAc, showed enhanced toxicity and Ac_4ManNAc could be considered to provide the "negative control" in these experiments.

DNA Ladder Assays—For DNA ladder assays, 1.0×10^6 cells were collected by centrifugation after ManNAc analog incubation and washed with phosphate-buffered saline. Cell pellets were resuspended in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 24 mM EDTA, and 0.5% SDS) containing 0.1 mg/ml proteinase K and then incubated at 55 °C overnight. DNA was cleared from the lysates by centrifugation and then extracted by using an equal volume of phenol/chloroform and precipitated by adding absolute ethanol and 0.3 M ammonium acetate at −20 °C overnight. The DNA was resuspended in sterilized water, treated with RNase A at 37 °C for 1.0 h, and then analyzed by gel electrophoresis on 2.0% agarose gel stained with ethidium bromide (0.5 μg/ml).

Phosphatidylserine Membrane Asymmetry Assays—For detection of apoptosis by Annexin V, 1.0×10^6 cells were incubated with analogs, collected by centrifugation, washed with Dulbecco's phosphate-buffered saline, and then suspended in binding buffer. The cells were then stained with FITC-labeled Annexin V and PI and analyzed by flow cytometry (32).

Assay of Caspase-3 Activity—After incubation with analog, 2.0×10^6 cells were collected and washed twice with Dulbecco's phosphate-buffered saline, suspended in lysis buffer, and then incubated on ice for 10 min, after which cell debris was removed by centrifugation and supernatants were used to determine protein content and enzyme activity. Total protein concentration was measured by total assay kit (Sigma) based on the modified Lowry's method. Samples were normalized for protein concentration and then added to a reaction buffer with 400 μM DEVD-ρNA and incubated for 1.0 h at 37 °C. The ρNA light emission was quantified using a spectrophotometer (Beckman) to determine absorbance values at 405 nm.

Analysis of the Expression of Sialoglycoconjugate-processing Enzymes—Total RNA was isolated from Jurkat cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA concentration was quantified spectrophotometrically, and equal amounts from each sample were used as templates for reverse-transcription PCR for first-strand DNA using Superscript RT II (Invitrogen). RNA integrity was confirmed using 18 S rRNA primers, and samples were standardized for equal levels of β-actin cDNA. PCR was performed for 30 cycles by the following program for each cycle: denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The primers used for the detection of human sialidase 1 were 5′-CAC TGC CAC AGG GGT ATT CT-3′ and 5′-TCT CAG ATG AGG GCA GGA CT-3′ (35). Primers used for the detection of α2,3-sialyltransferases were: 5′-GCA CTG TCA CAC CTC TGC AT-3′ and 5′-ACG TTG TCC CCA CTC AAG AC-3′ for Galβ1,3GalNAc α2,3-sialyltransferase (SIAT4A (36)); 5′-CAG GAG GTG GGA CAA CAC TT-3′ and 5′-TTT GGC GGC TTG AAA TAA TC-3′ for Gal β1,3GalNAc α2,3-sialytransferase (SIAT4B (37)); 5′-CTA GCC ATC ACC AGC TCC TC-3′ and 5′-GTG GGC AGA TTC AGG GTA GA-3′ for Galβ1,3GalNAc/Gal β1,4GlcNAc α2,3-sialyltransferase (SIAT4C (38)); 5′-CCT TTT GGG ATC AAA GGT CA-3′ and 5′-CGT CCC AGA GAC TTG TT-3′ for *N*-acetyllacosaminide α2,3-sialyltransferase (SIAT6 (39)); and 5′-CCC TGA ACC AGT TCG ATG TT-3′ and 5′-CAT TGC TTG AAG CCA GTT GA-3′ for CMP-NeuAc:lactosylceramide α-2,3-sialyltransferase (SIAT9 (40)). Primers used for the detection of α2,6-sialyltransferases were 5′-CGC CGG AGA GAA ATG AGT AG-3′ and 5′-CAG TGT CTT GTT GCC GAG AA-3′ for CMP-Neu5Ac GalNAc α2,6-sialyltransferase member VI (ST6 GalNAcVI (41)) and 5′-CTG CAG CTC ACC AGG ATG TA-3′ and 5′-TCC CAT AGA CCA CGA TCT CC-3′ for NeuAcα2,3Galβ1,3GalNAcα2,6-sialyltransferase (SIAT7D (42)). All primers were from MWG-Biotech (High Point, NC) and designed using the Primer3 software (43). Electrophoresis was performed on the PCR product in 1.8% agarose gels buffered with TAE buffer, and the result was visualized under UV illumination by ethidium bromide staining.

Experiments to Test Inhibition of Metabolic Flux

Analog Treatment of a "Sialuria" Jurkat Subline—Jurkat cells with feedback inhibition defects in GNE (the UDP-GlcNAc 2-epimerase/ManNAc 6-kinase bifunctional enzyme that regulates metabolic flux into the sialic acid pathway (44)) were obtained from a forward genetics scheme as described previously (33). These cells were incubated at an initial seeding density of 5.0×10^5 cells/ml, in the presence of each of the per-acetylated ManNAc analogs shown in Fig. 9 at concentrations up to 300 μM. On the third day of incubation, 100 μl of cells were removed and counted, and additional medium was then added to the remaining cells to reduce cell density to the initial seeding density of 5.0×10^5 cells/ml; sufficient analog was also added to maintain the original concentrations. After 5 days of incubation, cells were counted, and the sialic acid content was determined by the periodate resorcinol assay as described above.

Co-incubation of Wild-type Jurkat Cells with Ac_4ManNAc and the Panel of Analogs—Pre-mixed solutions of containing 100 μM Ac_4ManNAc (final concentration) and various concentrations of each of the analogs under test (up to 1.0 mM, final concentration) were prepared in tissue culture dishes, and then 5.0×10^6 Jurkat cells were added in complete medium to give a final volume of 5.0 ml. After 18 h of incubation, cells were counted, and sialic acid levels were determined by the periodate resorcinol assay.

RESULTS

Metabolic Production of Sialic Acid Is Modulated by the Structural Modification of ManNAc—Total cellular concentrations of sialic acid in cells treated with different sugars were determined by using the periodate-resorcinol assay to compare the metabolic conversion of ManNAc analogs to sialic acid. This assay was performed after cells were incubated with ManNAc analog for 2 days, which was previously determined as the length of time required to maximize sialic acid production (26). The ability of ManNAc analogs with hydroxyl and *N*-acyl struc-

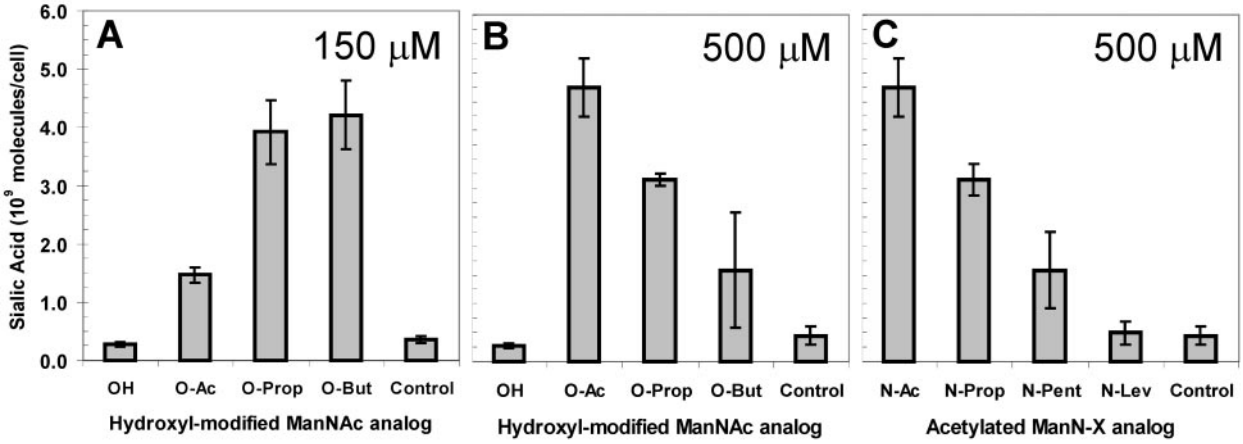

FIG. 2. **The effect of *N*-acyl (R_1) and hydroxyl (R_2) substitutions of ManNAc analogs on sialic acid production.** Total levels of cellular sialic acid were determined 48 h after the addition of 150 μM (*A*) or 500 μM (*B*) of hydroxyl-modified (R_2) analog to the culture medium of Jurkat cells; corresponding data for 500 μM *N*-acetylated R_1-modified analogs are shown in *C*. Data shown represent three to five replicate runs, and *error bars* represent standard deviation of the mean.

tural modifications to support sialic acid production in Jurkat cells is outlined in Fig. 2. At moderate levels of exogenous analog (150 μM, Fig. 2*A*) an increase in the number of carbon atoms in the ester derivatives attached to the hydroxyl groups of the sugars increased the metabolic efficiency of analog utilization. At the higher concentration of 500 μM (Fig. 2*B*), sialic acid production continued to increase for Ac_4ManNAc, as was anticipated from previous results (26), but decreased for $Prop_4$ManNAc and But_4ManNAc. The structure of the *N*-acyl side chain (where the *O*-hydroxyl group was held constant) also had a significant impact on metabolic efficiency because Ac_4ManNProp, Ac_4ManNPent, and Ac_4ManNLev each supported stepwise lower metabolic flux compared with Ac_4ManNAc, as shown in Fig. 2*C*.

Metabolic Production of Sialic Acid Is Correlated with Cell Viability for All ManNAc Analogs—Sialic acid production was next compared with the rate of cell growth and viability. Fig. 3, *A* and *D*, show the cell density for the 4-day period after hydroxyl- and *N*-acyl-modified analogs (respectively) were added to the culture medium of Jurkat cells seeded at 1.0×10^6 cells/ml. The final cell density of medium containing any of the ManNAc analogs was lower than for medium without analog, indicating that each of these modified sugars inhibited cell growth. The results shown in Fig. 3, *A* and *D*, are based on the direct enumeration of intact cells; a further refinement of these data by determining the fraction of viable cells (Fig. 3, *B* and *E*) revealed that the effects of different ManNAc analogs was more dramatic than initially portrayed by the cell counts. For example, Ac_4ManLev caused a dramatic reduction in cell viability after 1 day, whereas the other analogs did not evoke measurable toxicity until the second or third day. In general, the toxicity of each sugar analog increased as the number of carbon atoms increased, regardless of whether the modification was to the hydroxyl group (Fig. 3*B*) or to the *N*-acyl group (Fig. 3*E*). Finally, as shown in Fig. 3, *C* and *F*, for hydroxyl- and *N*-acyl-modified analogs, respectively, the production of sialic acid is correlated with cell viability. As reported previously for Ac_4ManNAc (26), the highest production of sialic acid occurs in rapidly growing, viable cells for each of the analogs now tested.

The Decreased Viability of Analog-treated Cells Is Due to Apoptosis—The cellular basis for the link between decreased metabolic flux through the sialic acid pathway and loss of cell viability was tested by using a set of complementary assays to demonstrate that decreased cell viability caused by ManNAc analogs is attributable to apoptosis.

DNA Fragmentation Assays—ManNAc analog-induced apoptosis was first demonstrated by DNA fragmentation, which is a biochemical hallmark of apoptosis. Fig. 4*A* shows typical DNA fragmentation in cells undergoing apoptosis induced by staurosporine, a compound that arrests cell cycle progression in a variety of cell types (45, 46), and at higher concentrations triggers both morphological change and intranucleosomal DNA fragmentation indicative of apoptosis (47). Similar DNA fragmentation was observed in cells treated with ManNAc analogs (Fig. 4*B*), indicating that ManNAc analogs also cause cell death by apoptosis. Not surprisingly, considering the higher toxicity of Ac_4ManNLev, this compound induced much higher levels of DNA fragmentation than seen for Ac_4ManNAc.

Phosphatidylserine Membrane Asymmetry Assays—To support the DNA fragmentation assays that identify late stages of apoptosis, we used Annexin V binding assays to detect loss of phospholipid membrane asymmetry and exposure of phosphatidylserine (PS) at the cell surface, which is an early event in the sequence of events that leads to apoptotic cell death. Fig. 5*A* shows the increase of fluorescence intensity generated by staining cells with PI, representing cell death, typically observed in cells treated with Ac_4ManNLev for 24 h. To identify whether this cell death was attributable to apoptosis, we used the fluorescence-labeled Annexin V, a Ca^{2+}-dependent, phospholipid-binding protein with high affinity for PS, to test whether PS was exposed on the cell surface. When analog-treated cells were double-stained with Annexin V, a large proportion (up to 79.11%) of the non-viable cells stained positive for PS (Fig. 5*B*), thereby supporting the DNA fragmentation results indicating that ManNAc analogs induce apoptosis.

Caspase Activation Assays—To further confirm the observation that ManNAc analogs induce apoptosis, we tested caspase activity. These enzymes play a critical role in the execution of apoptosis and are responsible for many of the biochemical and morphological changes associated with it (48, 49); consequently, caspase activity has been widely used to diagnose cells undergoing apoptosis. In this study, we confirmed that ManNAc analogs induced apoptosis by assaying the activation of caspase 3, one of the effector caspases. As shown Fig. 6, caspase 3 activity in cells treated with ManNAc analogs increased over a time period consistent with results from the DNA fragmentation and Annexin assays. Consistent with earlier results, caspase-3 activity was increased the most in cells treated with Ac_4ManNLev compared with other, less toxic analogs.

Expression of Sialogylcoconjugate-processing Enzymes—To provide a final piece of evidence that ManNAc analogs induce apoptosis, the expression of the genes coding sialyltransferases

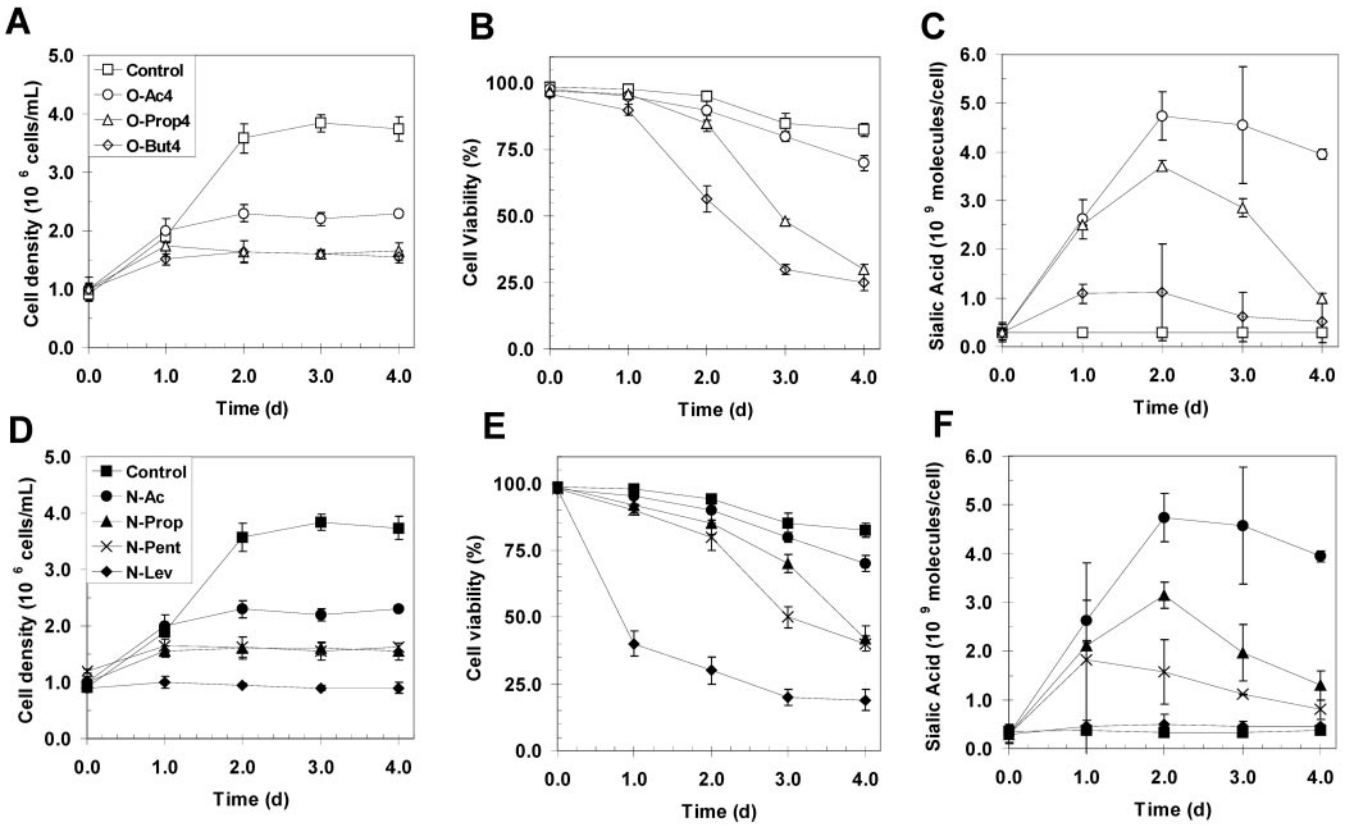

FIG. 3. **The effect of a panel of unnatural ManNAc analogs on cell growth, viability, and sialic acid production.** Jurkat cells were seeded at a density of 10^6 cells/ml and incubated in culture medium containing 500 μM of the indicated ManNAc analogs; in all cases, cells incubated without sugar analog were used as the control. The effects of hydroxyl modifications on growth, viability, and sialic acid production are shown in *A–C,* respectively; corresponding data are given for *N*-acyl modifications in *D–F*.

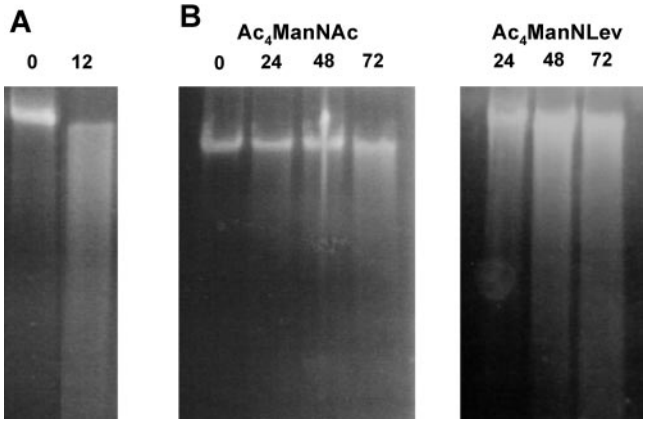

FIG. 4. **Agarose gel showing DNA fragmentation in cells undergoing apoptosis.** Jurkat cells were treated with 1.0 μM staurosporine (*A*) or 500 μM Ac_4ManNAc and Ac_4ManNLev (*B*).

and sialidases were monitored to test whether the link between the enzymes responsible for the surface expression of sialic acid and apoptosis observed in previous studies (50) held for ManNAc analog-induced apoptosis. First, cells were treated with staurosporine to induce apoptosis, total RNA was isolated, and RT-PCR was performed using specific primers for two sialyltransferases and a sialidase to confirm the alteration of gene expression in apoptotic cells reported by Azuma and co-workers (50). As shown in Fig. 7*A*, expression of sialidase increased in cells 1.5 h after treatment with staurosporine and then decreased noticeably within the next 1.5 h (*i.e.* at time = 3.0 h). Similarly, sialyltransferase expression decreased significantly within 3 h of staurosporine treatment; these results correspond with the rapid onset of apoptosis caused by this compound. The alteration of gene expression by Ac_4ManNAc analog treatment is next shown in Fig. 7*B* (*left panel*). The expression of sialidase and sialyltransferase in cells with treated with Ac_4ManNAc, which is the least toxic ManNAc analog under current evaluation, remained relatively constant during 96 h of incubation with this analog. This result showed that the increased flux through the sialic acid pathway supported by Ac_4ManNAc does not initiate apoptosis; rather, additional structural alterations to the ManNAc analog are required. One such alteration is the elongation of the ester substituents of the hydroxyl-modifying groups by two carbon units, such as the butyrate moieties of But_4ManNAc. In this case, expression of the ST3Gal III α2,3-sialyltransferase, the ST6GalNAc VI α2,6-sialyltransferase, and the sialidase under test were reduced to undetectable levels in a stepwise fashion at 24, 48, and 72 h, respectively (Fig. 7*B, center*), corresponding to a decrease in cell viability from ~90% to ~25% during this time (Fig. 3*C*). Cells treated with the highly toxic compound Ac_4ManNLev lost viability even more rapidly (within 24 h, Fig. 3*D*) and experienced a corresponding decrease in sialyltransferase and sialidase expression over the same time frame (Fig. 7*B, right panel*).

The two sialyltransferases tested in Fig. 7*B* are only a subset of the several varieties of these enzymes expressed in Jurkat cells. Therefore, to test whether these two enzymes show a representative response to ManNAc analog-induced apoptosis, the expression of a larger panel of sialyltransferases was monitored in Ac_4ManNLev-treated cells. As shown in Fig. 7*C*, all tested sialyltransferases were significantly down-regulated by 24 h, although the onset of the reduced expression occurred at different time points for individual enzymes. Moreover, certain sialyltransferases were transiently up-regulated before the characteristic apoptosis-associated down-regulation occurred.

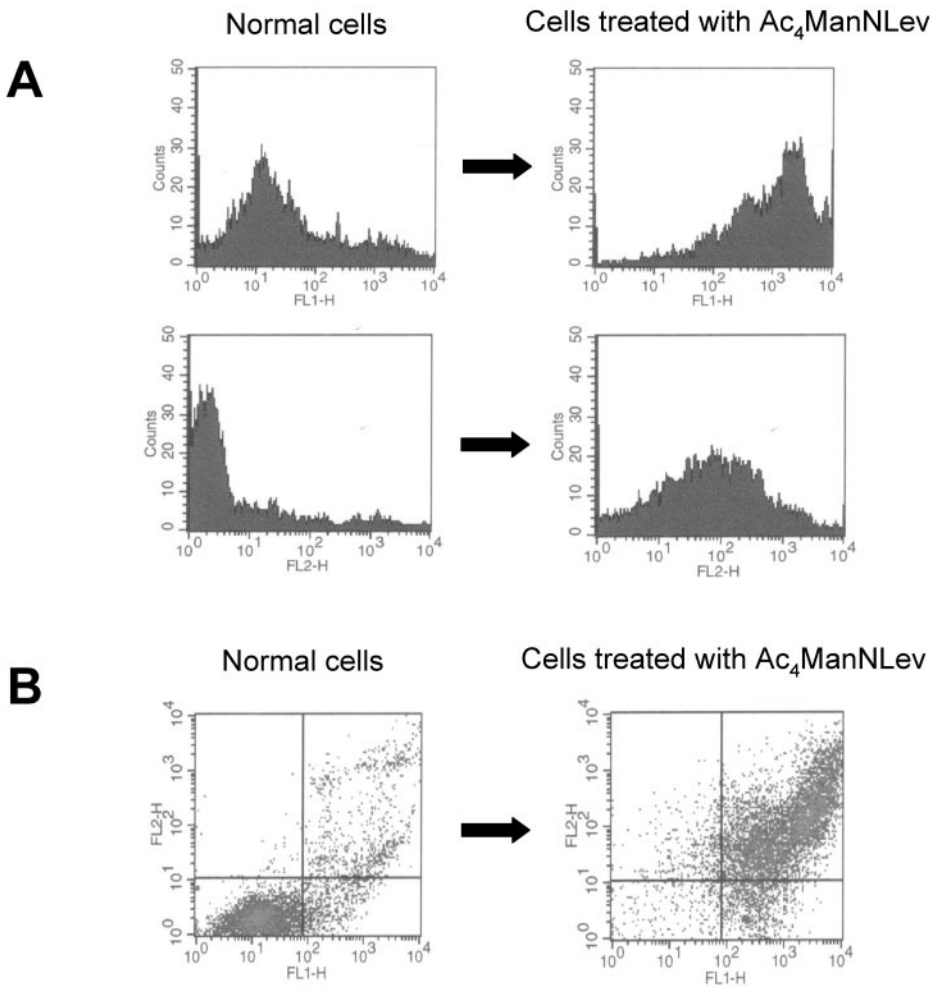

FIG. 5. **Flow cytometric analysis demonstrates that non-natural ManNAc analogs induce apoptosis.** Cells were treated with Ac_4ManNLev, collected after 24 h, stained with FITC-labeled PI and Annexin V, and then analyzed by flow cytometry (32). As shown in *A*, this treatment increased both PI (FL1-H) and Annexin (FL2-H) staining. Two-dimensional analysis (*B*) showed that the proportion of double-labeled cells increased from 12.79 to 79.11% upon analog treatment; this response is characteristic of apoptosis.

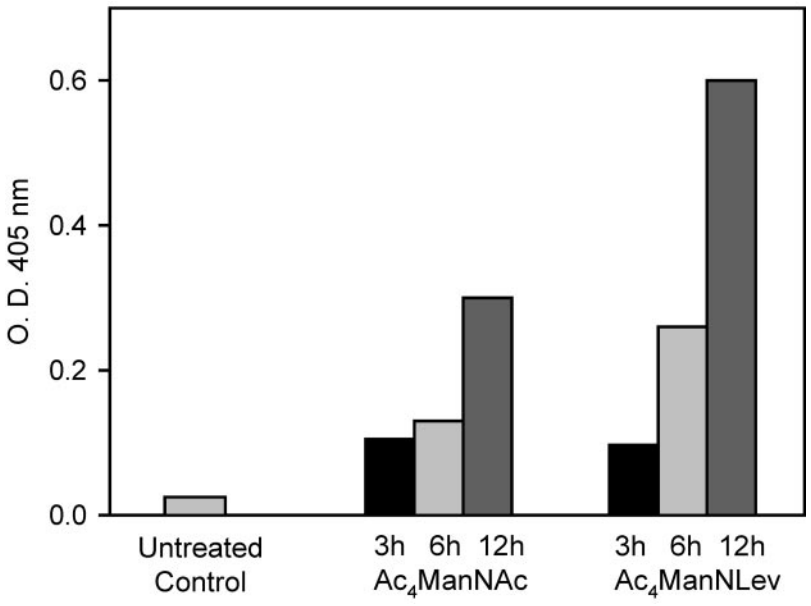

FIG. 6. **ManNAc analogs induce caspase-3 activity.** The activity of caspase-3 in cytosolic extracts prepared from Jurkat cells treated with 500 μM Ac_4ManNAc and Ac_4ManNLev for 3.0, 6.0, or 12 h and assayed by spectrophotometric detection of the chromophore tetrapeptide (DEVD-ρNA) after cleavage is shown.

The Precise Structure of the N-Acyl Group of ManNAc Determines Toxicity—An enlarged panel of *N*-acyl-modified ManNAc analogs (Fig. 9) was tested to further probe the structure-activity relationship that connects metabolic flux through the sialic acid pathway with toxicity; in particular, the number of carbon atoms in the *N*-acyl group and the exact position of the ketone in causing enhanced toxicity were tested. As observed previously for Ac_4ManNAc (26), the toxicity of each analog is dependent on cell density, making it difficult to assign exact quantitative comparisons of toxicity for each *N*-acyl modification (for example, Ac_4ManNHex is almost four times more toxic than Ac_4ManNAc when tested at a cell density of 6.25×10^4 cells/ml but only about 50% more toxic at 2.50×10^5 cells/ml and equally (non)toxic at 1.00×10^6 cells/ml). Regardless of the

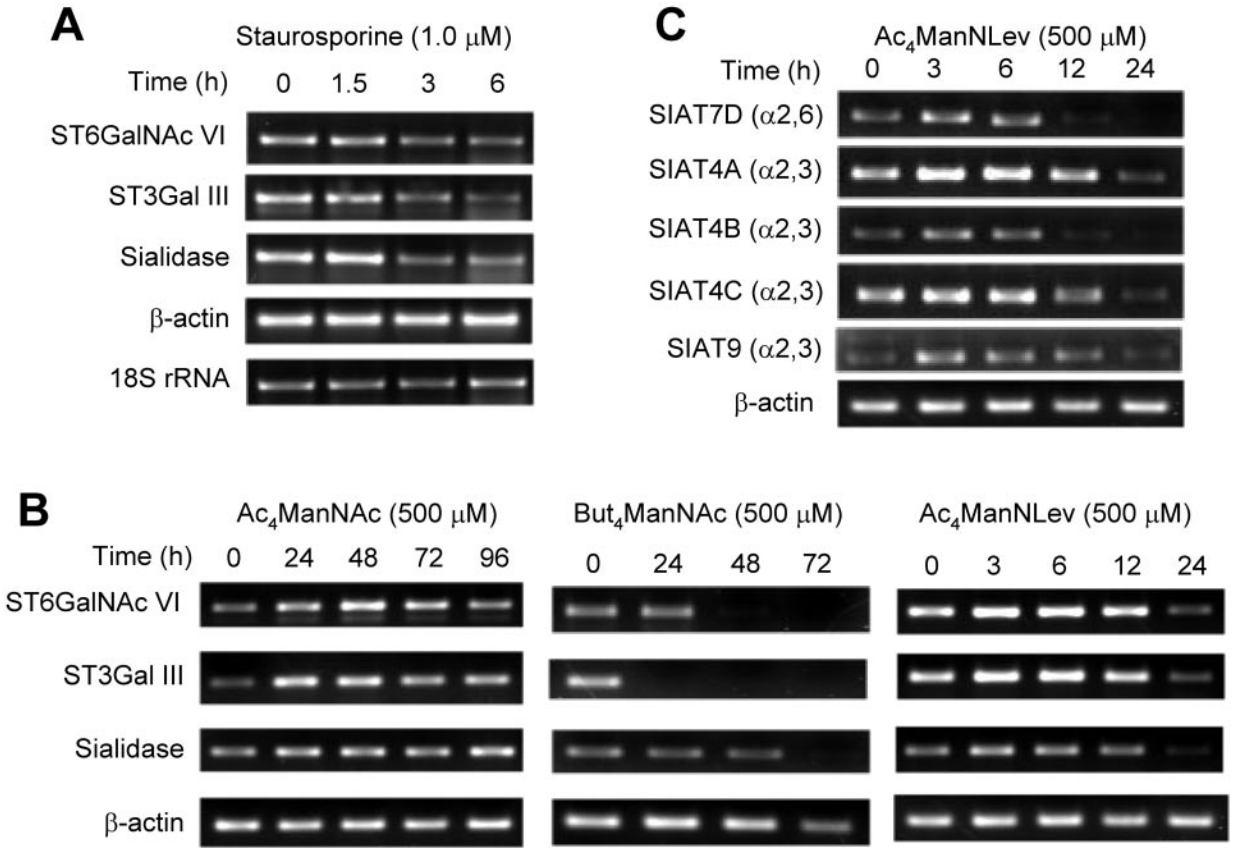

FIG. 7. **The alteration of gene expression in cells undergoing apoptosis.** Apoptosis was induced in Jurkat cells by incubation with (1.0 μM) staurosporine (*A*); Ac_4ManNAc, But_4ManNAc, or Ac_4ManNLev (*B*); and Ac_4ManNLev (*C*) for the number of hours indicated above the gel images.

exact comparative toxicities, two analogs, Ac_4ManNLev (cpd 6) and Ac_4ManNHomoLev (cpd 7), are clearly significantly more toxic than any of the other analogs tested. Interestingly, both of these analogs maintain a ketone in exactly the same position on the *N*-acyl group relative to the core mannosamine ring structure; the corresponding alkyl chain derivatives without ketones, Ac_4ManNPent (cpd 4) and Ac_4ManNHex (cpd 5), respectively, are much less toxic. Moreover, changing the position of the ketone group in relation to the core mannosamine structure by further elongation of the *N*-acyl moiety (cpds 8–10) ablates the high level of toxicity.

Inhibition of Metabolic Flux through the Sialic Acid Pathway Is Correlated with High Toxicity—Earlier in this study (Fig. 3) an inverse relationship was shown to exist between metabolic flux through the sialic acid pathway and toxicity. The molecular basis for this correlation, however, was not addressed until now. We raise the intriguing possibility that a direct link exists between metabolic flux through the sialic acid pathway and apoptosis in the case of *N*-acyl-modified analogs. As seen in Figs. 2 and 3, the highly toxic analog Ac_4ManNLev does not support a measurable increase in sialic acid production. At first, this correlation between toxicity and low flux does not appear to be significant because several additional analogs (Fig. 9; cpds 5, 8–10) with relatively low toxicity likewise do not measurably increase sialic acid production in wild-type Jurkat cells (data not shown). Upon closer consideration, however, the maintenance of sialic acid pathway intermediates (Fig. 8) at very low levels in wild-type cells may obscure important metabolic differences between the longer-chain ManNAc shown in Fig. 9. To explain more fully, previous work showed that ManNLev binds to one or more pathway enzymes with tight affinity but experiences low catalytic turnover (51) and may therefore not only not support increased flux through the pathway but may actually inhibit flux. However, intracellular levels of sialic acid in wild-type cells are too low to measure any further reduction upon inhibition of the pathway, and cell surface sialic acid levels are not sensitive to pathway inhibition under cell culture conditions because they can be supplemented by the scavenging of sialoglycoconjugates found in the serum (52).

To overcome these technical challenges, we took advantage of Jurkat cells harboring the same metabolic defect as found in sialuria (33, 53), specifically single amino acid mutations in the UDP-GlcNAc 2-epimerase/ManNAc 6-kinase (GNE) bifunctional enzyme (54), to test whether the highly toxic analogs Ac_4ManNLev and Ac_4ManNHomoLev inhibit metabolic flux (and, as a corollary, whether the less toxic analogs (cpds 5, 8–10 in Fig. 9) do not inhibit flux into the sialic acid pathway). In these cells, stringent feedback inhibition of GNE is lost because of weakened binding of CMP-sialic acid to the regulatory domain of this bifunctional enzyme (54, 55); as a result, sialic acid pathway metabolites increase to high levels, and total cellular sialic acid increases from $\sim 3.5 \times 10^8$ molecules/cell to $\sim 5.0 \times 10^9$ (33). These higher levels of intermediates provided an opportunity to test whether the excess flux through the pathway provided by the abnormally high GNE activity is inhibited by the highly toxic (or any other) analogs. When these cells were incubated with concentrations of each analog up to 300 μM, only Ac_4ManNLev and Ac_4ManNHomoLev reduced cell viability after 5 days of incubation (Fig. 10*A*). Importantly, these two compounds were also the only two sugars where sialic acid levels in cells actually decreased; other analogs either increased the already high levels (cpds 1–4) or had no effect on sialic acid levels (cpds 5,8–10) within a cell (Fig. 10*B*). In a supporting experiment, wild-type Jurkat cells were co-incubated with 100 μM

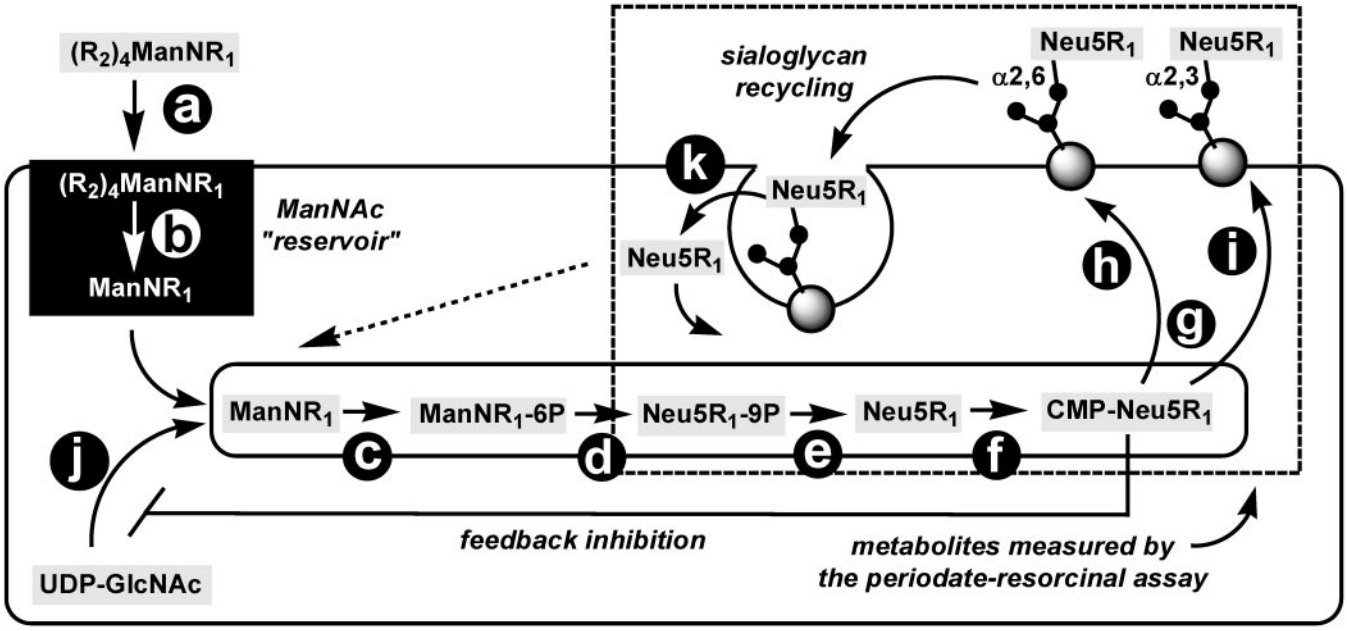

FIG. 8. **Outline of the sialic acid metabolic pathway.** ManNAc analogs enter a cell (*a*) and are stored in a "reservoir" (*b*) before entering the sialic acid pathway, which consists of the enyzmes ManNAc 6-kinase (*c*), sialic acid synthase (*d*), sialic acid 9-phosphatase (*e*), and CMP-sialic acid synthetase (*f*). These enzymes sequentially process ManNAc (or analog) into CMP-Neu5Ac (or analog), which is imported into the Golgi by the CMP-sialic acid transporter (*g*) and used in multiple parallel sialyltransferase reactions, such as the two shown (*h*, α2,6-sialytransferase or *i*, α2,3-sialyltransferase) to produce cell surface-displayed sialoglycoconjugates. This pathway is regulated by feedback inhibition via the binding of CMP-Neu5Ac to UDP-GlcNAc 2-epimerase, the enzyme that endogenously produces ManNAc from UDP-GlcNAc (*j*). Finally, cell surface sialoglycans are recycled and reused by a cell (*k*). It should be noted that all sialic acid-containing molecules detected by the periodate resorcinol assay used in this work are indicated in the *dashed box*.

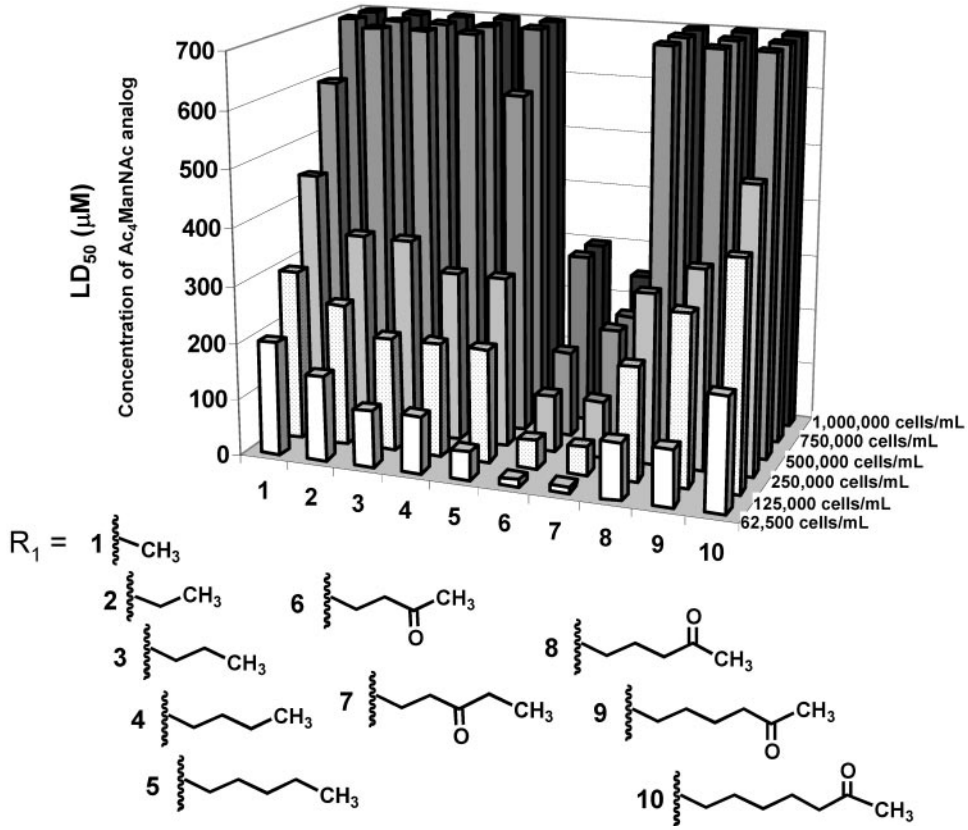

FIG. 9. **The toxicity of Ac_4ManNR_1 analogs is determined by cell density and the exact *N*-acyl modification.** Lethal dose values for Jurkat cells incubated with the panel of ManNAc analogs shown were determined by the method described by Jones and co-workers (26).

$Ac_4ManNAc$ and a range of concentrations of each analog over an 18-h time period (this shorter time frame was used to avoid the growth inhibition effects seen with the "sialuria" cells in Fig. 10*A*). In this experiment, the $Ac_4ManNAc$ included in all samples supported a baseline level of sialic acid production of $\sim 2.5 \times 10^9$ molecules/cell, thereby mimicking the increased flux of natural metabolites into the sialic acid pathway seen in "sialuria" cells. In this case, $Ac_4ManNLev$

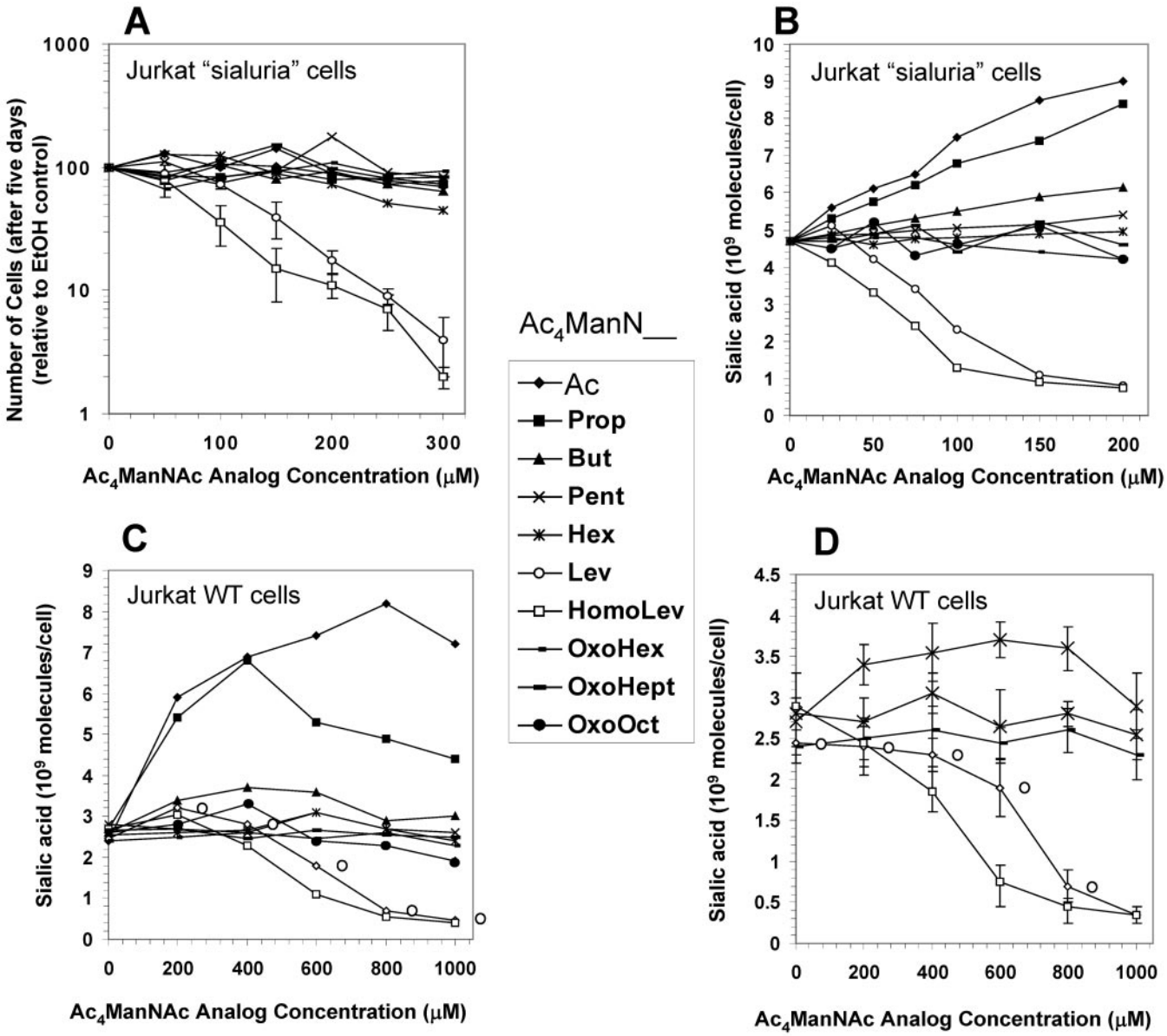

FIG. 10. **Inhibition of cell growth and metabolic flux through the sialic acid pathway depends on the exact position of the ketone group of the *N*-acyl group of Ac_4ManNAc analog.** Growth inhibition (*A*) and sialic acid production (*B*) after 5 days of incubation with the indicated concentrations of each ManNAc analog (delivered in the per-acetylated form) for a subline of Jurkat cells with the "sialuria" metabolic defect. *C*, sialic acid production for wild-type Jurkat cells incubated with 100 μM Ac_4ManNAc and the indicated concentrations (*x* axis) of each per-acetylated analogs is given after 18 h of incubation. *D*, The same data as indicated in *C* are given, but with a focus on structurally similar analogs, and with *error bars* (S.D.) provided to denote statistical significance (note that the error is of similar magnitude for each dataset shown in *C* but is omitted for clarity of the graph).

and Ac_4ManNHomoLev once again both inhibited flux (Fig. 10*C*), whereas the remainder of the analogs once again either increased flux (cpds 1–4) or had no measurable effect (cpds 5, 8–10).

DISCUSSION

The results presented in this report expand on previous work exploring the metabolic flux of non-natural ManNAc analogs through the sialic acid biosynthetic pathway. As discussed in more detail below, this work expands the repertoire of monosaccharide analogs available for glycosylation engineering applications by demonstrating that hydroxyl derivatives of increased chain length are used with high metabolic efficiency by a cell. In addition, this work has provided insights into the role of sialic acid in the complex sequence of events that occur during apoptosis; in several cases, the findings in this work set the stage for detailed future investigation into various aspects of the role of sialic acid in apoptosis.

In previous work, we demonstrated that fully acetylated ManNAc analogs are significantly (100–900-fold) more efficient than their free monosaccharide counterparts at supporting metabolic flux into the sialic acid pathway (26). This increased efficiency is believed to result from the hydrophobic properties endowed on the analog by the acetyl esters that facilitate passive diffusion of the compound into a cell (24, 27, 28). In the current work, we explored whether further extension of the ester-protecting groups would afford additional gains in metabolic efficiency by testing the ability of tetra-propanoylated ($Prop_4$ManNAc) and butanoylated (But_4ManNAc) analogs to support sialic acid biosynthesis. The success of this strategy, shown in Fig. 2*A*, demonstrated that the nonspecific esterases believed to remove the acetyl groups from non-natural sugars (24, 27, 28) are also

active on longer ester derivatives. This result shows that at moderate concentrations where these compounds are non-toxic, they offer an attractive alternative to acetyl-modified analogs for glycosylation engineering applications because they are used 2–3-fold more efficiently. However, when the concentrations of these analogs were increased from 150 to 500 μM, the level determined previously to support the highest level of flux for Ac_4ManNAc (26), sialic acid production decreased for $Prop_4$ManNAc and But_4ManNAc (Fig. 2*B*); furthermore, this drop-off in production was correlated with growth inhibition and loss of cell viability (Fig. 3, *A* and *B*). The fact that analog utilization is positively correlated with vigorous cell growth may hold important implications for the medical use of these analogs; for example, it could portend highly selective incorporation into rapidly growing cancer cells during analog-based treatment strategies (2, 11, 19, 20, 56).

Several complementary assays showed that growth inhibition and loss of cell viability observed in cells incubated with high concentrations of ManNAc analogs was attributable to the initiation of apoptosis by these compounds (57–59). These assays, which included DNA fragmentation analysis (Fig. 4), Annexin-FITC two-dimensional flow cytometry demonstration of phosphatidylserine exposure on the cell surface (Fig. 5), and caspase-3 activation (Fig. 6), confirmed that ManNAc analogs induced apoptosis in the Jurkat cells (similar effects have been confirmed in additional cell types; data not shown). Furthermore, ManNAc analog-induced apoptosis leads to early-onset changes in the expression of sialic acid-processing genes directly responsible for the display of sialic acid on the cell surface (Fig. 7). More specifically, sialidase is transiently up-regulated, which increases the rate of removal of sialic acid from sialoglycoconjugates during membrane recycling and sialyltransferases are down-regulated, thereby preventing the biosynthesis of new surface sialoglycoconjugates. Together, these factors reduce cell surface display of sialic acid in cells undergoing apoptosis; reduction in the sialic acid content of carbohydrate chains exposes penultimate galactose residues on surfaces of apoptotic cells and increases phagocytosis (60, 61).

As outlined in the previous paragraph, both hydroxyl- and *N*-acyl-modified analogs trigger several features universally associated with the apoptotic response; these findings, however, do not provide molecular level detail of the exact mechanism of apoptosis. One possibility is that these analogs enter the cytoplasm and serve as typical chemical toxicants and transmit apoptotic signals through the intrinsic mitochondrial apoptotic pathway (62). Another possibility is that hydroxyl-derivatized analogs are sequestered in cellular membranes that serve as a "reservoir" for these compounds (26). Upon saturation with analog, the biophysical properties of the membranes are altered sufficiently to inflict sufficient mitochondrial damage to initiate the intrinsic apoptotic response (note that analogs can be used without initiating deleterious cellular effects provided that capacity of the reservoir is not exceeded (26)). Alternately, we are testing whether membrane properties are changed sufficiently to impact surface receptors such as Fas or tumor necrosis factor-α that are able to initiate the receptor-mediated apoptotic pathway.

Regardless of the exact mechanism used by hydroxyl-derivatized ManNAc analogs to initiate apoptosis, the *O*-acyl groups are believed to be removed before the entry of analog into the sialic acid pathway (22, 24, 27), suggesting that analog-induced apoptosis is not attributable to changes in sialic acid metabolism. Closer analysis, however, indicates that a subset of analogs attains highly toxic properties through a direct connection to the sialic acid pathway. Evidence for this hypothesis is provided by analysis of the panel of *N*-acyl-modified ManNAc analogs shown in Fig. 9, where the exact position of a ketone functionality on the *N*-acyl group of a ManNAc analog is the key determinant of both toxicity (Fig. 9) and inhibition of metabolic flux into the sialic acid pathway (Fig. 10). It is important to note that only the two analogs (Ac_4ManNLev and Ac_4ManNHomeLev) that actually inhibit metabolic flux are highly toxic; analogs that increase flux (Fig. 9, cpds1–4) or have a negligible effect on flux (cpds 5,8–10) are much less toxic. One explanation for these effects is that a shut-down of early stages of sialic acid metabolism (see Fig. 8) initiates apoptosis. A second possibility is that increased metabolic flux into the sialic acid pathway can "rescue" cells during early stages of apoptosis (note that toxicity is lowest for Ac_4ManNAc and Ac_4ManNProp, the two analogs with the highest flux through the pathway); conversely, inhibition of flux may exacerbate apoptosis initiated by alternate pathways, thereby accounting for the high toxicity of Ac_4ManNLev and Ac_4ManNHomeLev.

The inhibition of sialic acid production by Ac_4ManNLev and Ac_4ManNHomoLev must occur at an early stage in the sialic acid pathway (Fig. 8) because, after these analogs intercept the pathway, they only require two enzymatic transformations to produce molecular species detectable in the periodate resorcinol assay (Fig. 8). The first step, phosphorylation of ManNAc on the C-6 position by the kinase activity of GNE, is unlikely to be affected, and even if it is inhibited, this step can be complemented by alternate cellular sources of hexosamine kinase activity. The next step, catalyzed by sialic acid synthase (63), is a more likely candidate for specific inhibition because the ketone of the highly toxic analogs may have the potential to occur in the correct spatial orientation to substitute for the phosphoenol pyruvate co-substrate normally used in this step (as based on modeling considerations, not shown). Experimental support regarding the inhibition of sialic acid synthase will be gained in future competition assays testing the ability of the recombinant enzyme (63) to catalyze the conversion of ManNAc 6-P to NeuAc 9-P in the presence of ManNLev 6-P, ManNHomoLev 6-P, or second-generation inhibitors based on these compounds.

The proposed link between a reduction in metabolic flux through the early stages of sialic acid pathway and apoptosis established in this work complements previous reports linking apoptosis with a reduction of mature sialoglycoconjugates (50). Together, these findings provide a coherent picture of the overall down-regulation of cell surface sialylation, a process that facilitates phagocytic uptake of apoptotic cells through the asialoglycan receptor (60, 61). In addition to the overall loss of surface sialic acid at a late stage of apoptosis, the reduction of specific forms of sialic acid during early stages may also contribute to apoptosis; for example, loss of sialic acid on Fas increases the sensitivity of the host cell to Fas-mediated apoptosis (30, 64).

The complete enunciation of the connections between intracellular sialic acid metabolism, sialoglycoconjugate display on the cell surface, and apoptosis is proving to be extremely complex, and many molecular details remain unexplained. For example, the reduction in surface sialic acid discussed above conflicts with findings where the presence of a sialic acid residue plays an active role in the induction of apoptosis. More specifically, the addition of a sialic acid residue to the ganglioside G_{M3} to form G_{D3} is sufficient to initiate apoptosis in several types of cells (6, 31), and increased sialylation of CD43 occurs during early stages of apoptosis, leading to capping of this glycoprotein and subsequent recognition and uptake of the affected cell by macrophages (29). The results shown in Fig. 7*C*, where transient up-regulation (or the delayed onset of down-regulation) occurs for certain sialyltransferases corresponds to previous reports that these enzymes are independently regu-

lated (65) and provides a mechanistic explanation for the different, and sometimes opposing, sialylation fates experienced by various sialosides during apoptosis. It should be noted, however, that the exact correspondence between individual sialyltransferases and specific cell surface molecules altered during apoptosis remains to be established.

In conclusion, many recent reports have established tentative links between sialic acid and apoptosis in a variety of human diseases including AIDS (66), viral and pathogen infection (67–70), heart disease (71), and cancer (64, 72), as well as in natural developmental and aging processes (31). Consequently, an enhanced understanding of the underlying biological basis of the many emerging roles of sialic acid in apoptosis is urgently needed. Toward this goal, ManNAc analogs used in sialic acid engineering applications are shown in this work to be able modulate specific aspects of sialic acid metabolism and therefore are valuable research tools to probe intricacies of the relationship between sialic acid and apoptosis.

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